MICROBIAL DESULFURIZATION MECHANISMS

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Key Words: Bacterial desulfurization, sulfur-specific degradation

ABSTRACT
Sulfur-specific desulfurization with *Rhodococcus rhodochrous* IGTS8 and related microorganisms may be a useful technology for upgrading high-sulfur carbonaceous materials to more environmentally acceptable fuels. Model compound studies with the organism showed that dibenzothiophene was converted to 2-hydroxybiphenyl or 2,2-dihydroxybiphenyl, depending on whether growth or nongrowth conditions were used in the desulfurization experiments. The pathways that lead to the two key intermediates in the degradation (2'-hydroxybiphenyl-2-sulfinic acid and 2'-hydroxybiphenyl-2-sulfonic acid) and the subsequent conversion of these intermediates to the phenol products required further understanding. The synthesis of an ^{18}O-enriched sulfone substrate has been accomplished, and experiments were performed to elucidate the mechanism of the conversion to the sultine intermediate.

INTRODUCTION

Microbial systems that desulfurize organosulfur compounds have potential use in removing sulfur from fossil fuels. Some organisms utilize a catabolic pathway that results in excision of the sulfur without converting organic carbon to carbon dioxide. Dibenzothiophene (DBT) has been useful as a model compound to investigate the sulfur-specific behavior in bacterial systems. Intermediates corresponding to this thiophenic-ring scission (4S) pathway have been isolated and characterized (1).

The desulfurization of DBT by *Rhodococcus rhodochrous* IGTS8 was recently demonstrated to proceed via two pathways that result in formation of either 2-hydroxybiphenyl or of 2,2'-dihydroxybiphenyl, depending on whether growth or nongrowth conditions are used in the desulfurization experiments (2). Under nongrowth conditions, the DBT was converted to 2'-hydroxybiphenyl via the 2'-hydroxybiphenyl-2-sulfinate (analyzed as the cyclic sultine ester form). Under growth conditions, very little of the 2'-hydroxybiphenyl-2-sulfinate was converted to 2-hydroxybiphenyl; instead, most was oxidized to 2'-hydroxybiphenyl-2-sulfonate (analyzed as the sultone form), and 2,2'-dihydroxybiphenyl was the major product. The oxidation of the sulfinate to the sulfonate occurs spontaneously (nonenzymatically) in aqueous buffer exposed to air, but the sulfinate may also be oxidized in an enzyme-catalyzed reaction. These pathways are summarized in Figure 1. Further understanding of the details of the pathways and mechanisms of the various steps in the pathways is needed.

The initial reaction in the 4S pathway is the oxidation of DBT to the sulfoxide (DBT-5-oxide) (Reaction A). Further oxidation to the sulfone (DBT-5,5-dioxide) also occurs. What is not known is whether the sulfoxide (Reaction B) or the sulfone (Reaction G) is the immediate precursor for the 2'-hydroxybiphenyl-2-sulfinate under nongrowth conditions and whether the sulfone can be converted directly to 2'-hydroxybiphenyl-2-sulfonate (Reaction E) under growth conditions. Is the sulfone an intermediate in the 4S pathway or a by-product?

When sulfone was fed to the bacterium under nongrowth conditions, 2'-hydroxybiphenyl-2-sulfinate and 2'-hydroxybiphenyl were produced (2). Since the sulfone is reduced back to the sulfoxide, whether the sulfoxide or the sulfone is the precursor of 2'-hydroxybiphenyl-2-sulfinate could not be distinguished in the earlier experiments.

This paper describes the use of ^{18}O-enriched sulfone (DBT-5,5-dioxide-$^{18}O_2$) to elucidate the pathway involving the sulfone. If prior reduction of the sulfone to sulfoxide occurred in the pathway (reverse of Reaction D), then loss of one labeled oxygen would be observed in the sultine and sultone intermediates.

RESULTS AND DISCUSSION

The ^{18}O-labeled DBT sulfone substrate (98% isotopic purity) was utilized in the desulfurization experiment with the bacterium *Rhodococcus rhodochrous* (ATCC #53968) under nongrowth conditions. The supernatant from centrifugation of the culture medium was extracted with ethyl acetate, and gas chromatography (GC) analysis showed only starting sulfone and final product, 2-hydroxybiphenyl, and no sultine (cyclic form of the sulfinate) was present. From experiments with unlabelled sulfone, we know that all the sulfinic acid intermediate present in the culture medium is present in the open sulfinate form, but cyclizes to the sultine when the pH is lowered. The sultine can then be easily extracted with ethyl acetate and analyzed by GC. Therefore, the aqueous layer obtained

after the initial extraction (from the experiment with ^{16}O-enriched substrate) was acidified and again extracted. GC/mass spectrometry (MS) was performed to obtain the isotopic abundances for the peaks corresponding to the sultine and sultone.

It must be pointed out that the cyclization to the sultine or sultone form displaces one oxygen from the sulfur, thus half of the label will be eliminated from each molecule of sultine. Hence, the sultine molecules will contain a single labeled oxygen if Reaction G is followed. If the pathway involving Reaction B is followed, half of the sultine molecules will be single labeled and half unlabeled, as a result of the oxygen displacement. These projections assume no occurrence of exchange or disproportionation, which would decrease or increase the numbers of labeled oxygens on sulfur.

The mass spectrum of the sultine product showed molecular ions at m/e 218 and 216 (corresponding to single-labeled and unlabeled species, respectively) in the ratio 2.7. Since this greatly exceeds the ratio of 1 predicted for Pathway B, the ratio is consistent with Reaction G, and the much smaller number of unlabeled molecules must have been formed by exchange with water or disproportionation. The sultine mass spectrum shows a large fragment ion at m/e 190, resulting from loss of $C^{16}O$, and no peak at m/e 188, corresponding to loss of $C^{18}O$. Therefore, all the ^{18}O must be attached to the sulfur, and none is on the phenolic carbon.

Thus, the pathway must proceed directly from the sulfone to the sulfinate (**Reaction G**) rather than reducing sulfone back to the sulfoxide intermediate. The reaction that forms the sulfinate from the sulfone is actually a reduction with respect to the sulfur. When the carbon–sulfur bond is cleaved, the electrons flow to the sulfur to form the sulfinate anion.

The cleavage reaction that involves formation of the sulfinate is similar to that in the electrochemical (3) and metal reduction of sulfones (4), except that hydroxyl is introduced at one aryl site in bacterial reaction, whereas hydrogen is added in the electrochemical and metal reduction reactions. The reactions of DBTs with basic reagents, such as KOH, have been investigated. Attack of nucleophilic oxygen was reported to occur at the ring carbon at high temperatures in the presence of crown ether, resulting in the formation of 2'-hydroxybiphenyl-2-sulfinate (5). In the absence of crown ether, very small amounts of the sulfinate were formed, and attack at the sulfur occurred (5, 6).

In the bacterial system, the initial stage of the mechanism is more likely to involve addition of oxygen to the benzene ring via a hydroperoxyflavin cofactor. This type of mechanism has been described for oxygenase reactions, such as the salicylic acid and *p*-hydroxybenzoic acid oxygenase reactions that occur in other bacteria.

Formation of the sulfonate anion by simple addition of oxygen to the sulfinate anion would not be expected to change the number of labeled oxygens on the sulfur, and the sulfonate would be expected to be double-labeled. Cyclization of the sulfonate to the sultone involves loss of one of the oxygens on the sulfur. Thus one-third of the sultone would be double-labeled and two-thirds would be single-labeled. But the sulfinate oxidation reaction might be more complex, and the labeling will provide some information on this oxidation.

The mass spectrum of the sultone exhibited the molecular ion peaks at 236, 234, and 232 in the ratio 5.6:3.2:1. Thus the sulfonic acid corresponding to the sultone must have been primarily triple-labeled. The obvious explanation for this fact is that the sulfinate undergoes a dimerization reaction and subsequently transfers labeled oxygen between sulfur atoms as the sulfonic acid is formed (7). The sulfur that lost oxygen in the reaction of the dimer may end up, after subsequent oxidation, as part of the unlabeled sultine that was observed.

The results not only prove that the sulfone is directly converted to the sulfinate, but also that the oxidation of the sulfinate to sulfonate follows accepted chemical mechanisms. A pathway involving Reaction E is therefore very unlikely to occur, since it would not produce triple-labeled sultone product.

The results would be more straightforward if the sulfinate were analyzed by conversion to a derivative that preserves both labeled oxygens attached to sulfur. The methylsulfone derivative was prepared by treating the sulfinate with methyliodide, and analysis of this derivative will be used to confirm the findings from the sultine derivative.

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ACKNOWLEDGMENTS

This work was supported in part by the U.S. Department of Energy (Contract No. DE-FC21-93MC30097). The assistance of David J. Miller is gratefully acknowledged.

FIGURE 1